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(בעברית) (Hebrew)

PHOTOREACTIVE COMPOUND SPECIFICALLY BINDING TO CALCIUM BINDING PROTEINS
(באנגלית) (English)

מבקש בזאת כי ינתן לי עליה פטנט hereby apply for a patent to be granted to me in respect thereof.

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PHOTOREACTIVE COMPOUND SPECIFICALLY BINDING TO CALCIUM BINDING PROTEINS

PHOTOREACTIVE COMPOUND SPECIFICALLY BINDING TO CALCIUM BINDING PROTEINS

Field of the Invention

The present invention relates to a photoreactive reagent that binds specifically to calcium-binding proteins, links to them covalently after photo-activation, and thus labels them and enables their characterization and purification. The invention also provides methods for the preparation of affinity chromatography matrix and protein biosensors comprising said reagent, and relates to therapeutic and diagnostic uses of said reagent and biosensors.

Background of the Invention

Calcium (Ca^{2+}) is a ubiquitous intracellular signal transducer, responsible for controlling numerous cellular processes. It regulates muscle contraction, neurotransmitter release, hormone secretion, cell motility, cell mitosis, and gene expression. Ca^{2+}-signaling is employed throughout the life of the organism, beginning with a surge of Ca^{2+} at fertilization and ending with the induction of apoptosis, thereby, at cell death. Calcium is deeply involved in the cell proliferation. Several drugs that block Ca^{2+} entry into the cell, retard the cell growth in human melanoma, lung and colon carcinoma, and prostate cancer [See, e.g., Haverstick, D.M. et al.: Cancer Res. 60 (2000) 1002-8].

There are many proteins involved in the compound role of calcium in the organism having high-affinity or low-affinity Ca^{2+}-binding sites. Many of said proteins remain unknown or insufficiently characterized. Despite advances in defining Ca^{2+}-dependent activity, considerable experimental difficulties still remain in localizing the protein Ca^{2+}-binding sites [Hadad N. et al.: J. Biol. Chem. 269 (1994) 26864-9]. Fundamental to the understanding of normal and abnormal calcium signaling is the knowledge of the proteins involved in this process. It is therefore an object of this invention to provide a compound binding specifically to calcium-binding proteins.

It is an object of this invention to provide a photoreactive azido-ruthenium (AR) based probe which specifically binds to calcium-binding proteins, and inhibits their activity.

It is another object of this invention to provide an AR-based probe which specifically and covalently binds to Ca^{2+}-binding sites of calcium-binding proteins, thereby allowing to localize and to characterize such sites.

It is another object of this invention to provide the use of said AR-based compound in producing affinity chromatography matrices binding specifically Ca^{2+}-binding proteins, and allowing their purification.

It is still another object of this invention to provide the use of said AR-based compound in producing biosensors binding specifically to Ca^{2+}-binding proteins, and allowing their characterization.

It is further an object of this invention to provide diagnostic and therapeutic uses of azido-ruthenium compound associated with defects in the function of a Ca^{+}-binding proteins.

Summary of the Invention

This invention provides a new photoreactive azido-ruthenium compound, which exhibits a specific interaction with Ca^{2+}-binding proteins. The reagent, azido-ruthenium of this invention (AR), was found to specifically inhibit activities of various Ca^{2+}-transport mediating proteins, Ca^{2+}-regulatory proteins, and Ca^{2+}-dependent enzymes. When photo-activated, the reagent binds covalently to the calcium binding site. Azido-ruthenium according to this invention can be synthetized from radiolabeled reagents, which enables to label binding sites in calcium binding proteins, and to characterize these important proteins. The compound comprises ruthenium and azide in the molar ratio of 2:1. Said AR compound binds covalently after photo-activation by UV irradiation to the calcium-binding site of said protein. The binding is specific for calcium-binding proteins. In a preferred embodiment of this invention, the compound contains a radioactive isotope, preferably ^{103}Ru, and can label radioactively the

binding sites in said calcium binding proteins. The compound of this invention can bind to calcium-binding proteins, and inhibit their activity. Among the proteins which can bind the compound of this invention are calcium binding proteins selected from proteins involved in signal transduction, muscle contraction, neurotransmitter release, hormone secretion, cell motility, apoptosis, fertilization, cell proliferation, cell mitosis, gene expression: where some of these proteins are associated with mediating Ca^{2+} transport, such as Ca^{2+}-pump, the mitochondrial uniporter, channel protein VDAC (voltage dependent anion channel), Ca^{2+} release channel/ryanodine receptor, proteins involved in Ca^{2+} uptake in mitochondria, and the Ca^{2+} pump catalyzing the transport of Ca^{2+} across the sarcoplasmic reticulum membrane. When the compound of this invention is photo-activated after its binding to a protein by UV-irradiation, it binds covalently and thus inhibition of the activity of said protein increases several-fold, compared to the inhibition without photo-activation.

The invention also relates to the method of purifying, identifying, and characterizing calcium-binding proteins by employing AR compound containing ruthenium and azide in molecular ratio of 2:1. When studying function and structure of calcium-binding proteins, the method of this invention may be combined with methods known in the art, such as SDS-PAGE, autoradiography, MALDI-TOF analysis, LC-MS/MS, protein

sequencing, and a sequence homology search. In a preferred embodiment, this invention provides new affinity chromatography technique, comprising binding AR to particles of porous polymer that are packed in a column, wherein calcium-binding proteins are retained in said column, while other proteins pass. Said retained proteins are released by calcium ions. Said particles may comprise agarose, cellulose, or other matrix. Said method of this invention may comprise purifying a Ca^{2+}-binding protein, identifying an unknown Ca^{2+}-binding protein, inhibiting a Ca^{+}-binding protein, and labeling a Ca^{2+}-binding protein. Said labeling may be a radioactive labeling. In a preferred embodiment, the method of this invention comprises the preparation of an affinity resin. In another preferred embodiment, the method according to this invention comprises the preparation of a bio-sensor chip.

The invention provides a process for preparing a photoreactive azido-ruthenium reagent that binds to calcium-binding proteins, which comprises i) reacting in dark of sodium azide with ruthenium (III) chloride in the presence of HCl, ii) applying the reaction mixture of the previous step onto a chromatographic column, e.g. cation-exchange or hydrophobic interaction column, iii) collecting the fractions containing AR, wherein such fractions may be identified, e.g., according to the absorbance at 290 nm, iv) drying the collected fractions, and optionally dissolving said dried fractions and applying them again on a chromatographic column, and

drying obtained AR reagent from methanol, while eventually crystallizing it. In the process of this invention, HCl has preferably the concentration in the range from 0.5 mol/l to 2 mol/l, and sodium azide and ruthenium chloride react at about 100°C for about 2 to 4 hrs. This invention further provides a compound of empirical formula $Ru_2N_3Cl_5.5H_2O$, and solvates thereof, e.g. solvates further comprising H_2O and HCl.

This invention is also directed to the use of AR compound containing ruthenium and azide in a molar ratio of 2:1 in diagnosing disorders associated with Ca^{2+}-binding proteins, and in preparing a medicament for treating said disorder. The invention also relates to a pharmaceutical composition containing said AR compound, or a solvate thereof, and possibly a carrier, stabilizer, adjuvant, diluent, or excipient. Said AR compound, or a composition containing it, may be used in the preparation of a medicament for treating or preventing a disorder associated with a defect in the function of a Ca^{2+}-binding protein, wherein said preventing and treating comprise the inhibition of Ca^{2+}-binding proteins by said AR compound.

Brief Description of the Drawings

The above and other characteristics and advantages of the invention will be more readily apparent through the following examples, and with reference to the appended drawings, wherein:

Fig. 1. shows the purification of AR on Sephadex LH-20 column;

Fig. 2. shows the characterization of AR by TLC;

Fig. 3. shows UV spectra of AR, ruthenium red, $RuCl_3$, and NaN_3;

Fig. 4. shows IR spectrum of AR;

Fig. 5. demonstrates the inhibition by AR of the Ca^{2+}-pump activity in skeletal muscle membranes;

Fig. 6. shows the effect of AR on calcium uptake and electron transport in isolated mitochondria;

Fig. 7. shows the inhibition of ryanodine binding to its receptor by AR;

Fig. 8. shows the inhibition of VDAC channel activity by AR;

Fig. 9. illustrates possible identification of Ca^{+2}-binding proteins using [^{103}Ru]AR;

Fig. 10. illustrates the use of AR in affinity chromatography; and

Fig. 11. shows SDS-PAGE of Ca^{2+}-binding proteins isolated by AR-affinity chromatography.

Detailed Description of the Invention

This invention provides a new azido-ruthenium, which is photoreactive, and exhibits a specific interaction with Ca^{2+}-binding proteins. Synthesis of three azido-ruthenium compounds were reported [Seok W.K. et al.: J.Organomelallic Chem. 559 (1998) 65-71; Siebald H.G.L. et al.: Polyhedron 15 (1996) 4221-5; Vrestal J. et al.: Collection Czechoslov. Chem. Commun. 25 (1960) 2155-60]. Effects of these reagents on biological

activities, or their photoreactivities, were not described. Another known ruthenium reagent, ruthenium red, is not photoreactive. The preferred reagent of this invention can be prepared by reacting sodium azide with ruthenium chloride (III) and purifying the product on a chromatographic column, e.g. cation-exchanger or hydrophobic. Chromatography purification methods, useful for this purpose comprise, for example, CM-cellulose or Sephadex LH-20 columns. IR spectrum of the novel azido-ruthenium (AR) product shows the characteristic absorption of the bound azido group at 2072 cm^{-1}. It was surprisingly found that the azido-ruthenium compound of this invention interacts with Ca^{2+}-binding proteins and inhibits their activity. When photo-activated, by UV-irradiation, the reagent binds covalently to the calcium binding site. The effect of the reagent of this invention on activities of various proteins, including channels, receptors and enzymes, were tested, and it was found by us that the interaction is specific for the calcium binding proteins (Table 1). Azido-ruthenium according to this invention can be synthetized from radiolabeled reagents, thus enabling labeling of binding sites in calcium binding proteins. Such labeling enables discovering still unknown proteins, explaining still unclear aspects of regulations and signal transduction pathways depending on calcium ions, separating and characterizing the involved peptides and proteins, as well as determining their structure and function.

In one embodiment, to identify Ca^{2+}-binding proteins, proteins labeled by radiolabeled reagents are separated by SDS-PAGE, identified by autoradiography, cut from the gel and exposed to cleavage conditions. The degradation products are separated by a second SDS-PAGE, and the Coomassie stained labeled bands are sequenced to identify the Ca^{2+}-binding site. The labeled protein bands can be cut from electrophoresis gels, and subjected to MALDI-TOF analysis or LC-MS/MS, and the identity of the protein can be determined by a sequence homology search.

Above mentioned approach can be used, for example, for identifying mitochondrial systems for transporting Ca^{2+}, like ruthenium red-sensitive uniporter, Na^+ or H^+ dependent efflux mechanisms, and the rapid mode of uptake transporter.

Our results show that azido-ruthenium of this invention specifically inhibits the activity of proteins which mediate Ca^{2+} transport such as the muscle Ca^{2+}-pump, the mitochondrial uniporter, and the channel protein VDAC. An AR compound according to this invention also inhibits the activity of proteins possessing regulatory Ca^{2+}-binding sites such as the muscle Ca^{2+} release channel/ryanodine receptor. In contrast to that, azido-ruthenium has no significant effect on Ca^{2+} independent enzymatic reactions.

Using radioactive AR, Ca^{2+}-binding proteins can be identified, purified and their functions can be established, e.g., autoradiography helps to localize suspected proteins. In a preferred embodiment, the radioisotope used for the synthesis of AR is ^{103}Ru.

In another preferred embodiment, the AR reagent of this invention is used for separation of Ca^{2+}-binding proteins by affinity chromatography, wherein the AR reagent is covalently attached to an inert support filled in chromatographic column. The support can be, for example, a porous polymer, such as agarose, cellulose, or dextrane, etc.. A protein mixture is loaded into the column, Ca^{2+}-binding proteins are reversibly retained while other proteins pass. The retained proteins may be released, for example, by a calcium containing buffer.

In another embodiment of this invention, AR is coupled to polysaccharides, and is used for construction of unique and novel bio-sensor chips for isolation and identification of Ca^{2+}-binding proteins. In a preferred embodiment, this invention enables to identify proteins that have affinity for Ca^{2+}-binding proteins, i.e. proteins that are not necessarily calcium binding themselves, but interact with Ca^{2+}-binding proteins, forming elements of the signal transduction pathways, often elements that are difficult to detect and identify. In a preferred embodiment of this invention, surface plasmon resonance (SPR) is used for

detecting intermolecular interactions. The chip of this invention can be used, for example, for identifying new Ca^{2+}-binding proteins, or sites of interaction; binding affinity and kinetics, as well as epitope map can be also obtained.

Based on the above findings, the present invention is also directed to a pharmaceutical composition for use in inhibiting calcium dependent proteins involved in various disorders associated with Ca^{2+}-binding proteins and defects in these proteins.

The invention provides a process for preparing an azido-ruthenium compound, having the ratio Ru:N_3 of 2:1, that specifically binds to, and inhibits, calcium-binding proteins, comprising the steps of :

i) reacting in dark of sodium azide with ruthenium (III) chloride in the presence of HCl, ii) applying the reaction mixture of the previous step onto a chromatographic column, e.g. cation-exchanger or hydrophobic, iii) collecting the fractions which contain the required product, possibly identifying such fractions by measuring absorbance; and optionally iv) steps of drying the collected fractions, redissolving them, rechromatographing them, and drying said compound from methanol, eventually crystallizing it. The process of this invention provides an AR product that migrates as a single spot with Rf being about 0.9 during TLC on cellulose F plates, using 0.16 M ammonium formate, pH 8.5 and 20%

methanol, said product having an absorbance maximum at about 290 nm, and its absorbance is preferably about from 250 to 450 in a water solution, at a concentration of 1 mg/ml, usually said absorbance is about 350.

Thus, this invention provides a novel tool to identify, purify and characterize Ca^{2+}-binding proteins, and to sequence the Ca^{2+}-binding sites. In addition, the requirement for Ca^{2+} in a protein reaction or in the regulation of its activity can be tested. The invention may be useful as a pharmaceutical agent to monitor the involvement of Ca^{2+} in the activity or regulation of proteins. The invention can be used as well for the detection of defected Ca^{2+}-binding proteins in certain diseases and disorders.

The invention will be further described and illustrated in the following examples.

Examples

Materials and general methods

Chemicals

ATP, CM-cellulose, lactate dehydrogenase, glutamate dehydrogenase, alkaline phosphatase, NAD+, NADH, EGTA, EDTA, Tris, MOPS, NaN_3 and $RuCl_3$ were obtained from Sigma. [^{3}H]Ryanodine (60 Ci/mmol) and $^{45}CaCl_2$ were purchased from New England Nuclear. Unlabelled ryanodine

was obtained from Calbiochem. Ruthenium red (98% pure) was from Fluka. Sephadex LH-20 was obtained from Amersham Biosciences.

Preparations

Sarcoplasmic reticulum (SR) membranes were prepared from rabbit fast twitch skeletal muscle as described by Saito [Saito et al.: J.Cell Biol. 99 (1984) 875-85]. Mitochondria were isolated from rat liver as described by us previously [Gincel D. et al.: Biochem J. 358 (2001) 147-55]. VDAC was purified by a method developed in our laboratory, using columns of hydroxyapatite and reactive red agarose [Gincel D. et al.: J. Bioenerget. Biomembr. 32 (2000) 571-83].

Calsequestrin was isolated from rabbit skeletal muscle sarcoplasmic reticulum membranes as described by Cala S.E. et al. [J. Biol. Chem. 258 (1983) 11932-6]. Calmodulin was isolated from sheep brain as described by Gopalakrishna R. et al. [Biochem. Biophys. Res. Commun. 104 (2) (1982) 830-6]. Troponin was isolated from rabbit skeletal muscle as described by Potter J.D. [Methods in Enzymol. 85 (1982) 241-65].

Assays

Protein concentrations were determined by the standard Lowry procedure [Lowry O.H. et al: J. Biol. Chem 193 (1951) 224-265]

For the determination of [^{3}H]ryanodine binding, SR membranes were incubated for 20-60 min at 37°C in a standard binding solution containing 1M NaCl, 20 mM MOPS (pH 7.4), 50 µM free Ca^{2+} and 20 nM [^{3}H]ryanodine. Unbound ryanodine was separated from protein-bound ryanodine by vacuum filtration of the sample through nitrocellulose filters (0.3µm), followed by two washes with 4 ml ice-cold buffer containing 0.2 M NaCl, 10 mM MOPS (pH 7.4) and 50 µM $CaCl_2$. The retained radioactivity in the dried filters was determined by liquid scintillation counting. Specific binding of [^{3}H]ryanodine was defined as the difference between the binding in the presence of 20 nM [^{3}H]ryanodine and in the presence of 20 µM unlabeled ryanodine.

Ca^{2+}-accumulation in freshly prepared mitochondria (0.5 mg/ml) was assayed for 1 to 20 minutes at 30°C in the presence of 225 mM mannitol, 75 mM sucrose, 120 µM $CaCl_2$ (containing $3x10^4$ cpm/nmol $^{45}Ca^{2+}$), 5 mM HEPES/KOH pH 7.0, and 5 mM succinate with 0.1 mM Pi, or 4 mM $MgCl_2$ with 3 mM ATP. The Ca^{2+}-uptake was terminated by rapid Millipore filtration, followed by a wash with 5 ml of 0.15 M KCl. The Ca^{2+}-accumulation in SR was determined as described previously [Shoshan-Barmatz V. & Shaineberg A.: Biochim. Biophys. Acta 1065 (1991) 82-8]. Electron transport from succinate to cytchrome c was described in above cited article [Gincel D. et al.: J. Bioenerget. Biomembr. 32 (2000) 571-83].

Reconstitution of purified VDAC into planar lipid bilayer (PLB), single channel current recording, and data analysis were carried out as described in above mentioned publication.

Example 1

Azido-ruthenium synthesis

All steps were carried out in the dark. $RuCl_3$ (29.8 mg, 0.144 mmol) and NaN_3 (6.24 mg, 0.096 mmol) were dissolved in 1N HCl (2 ml), silted and incubated at 100°C for 3 hrs. The sample was then applied to a Sephadex LH-20 column (1.5 cm x 12 cm) pre-equilibrated with water (Fig.1). Free Ru^{3+}, but not the product, remained bound to the column matrix. The absorbance at 290 was measured and the peak was collected, lyophilized and analyzed by TLC on cellulose F plates using 0.16 M ammonium formate, pH 8.5 and 20% methanol as a developer. The product migrated as a single spot with Rf = 0.9 (Fig. 2). The product is soluble in water, DMF and DMSO, less soluble in methanol, and insoluble in ethanol, ether, chloroform, ethyl acetate, n-butanol, and isopropyl alcohol. The purified product of azido-ruthenium has a maximal absorbance at 290 nm, the absorbance of a solution of 1 mg/ml being about 35. The spectrum of the product is different from that of the substrates and of ruthenium red, a known inhibitor of calcium dependent activity, which is not photoreactive (Fig. 3). Infrared spectrum of the product indicates the presence of a specific peak representing a bound azido group (Fig. 4). For further

purification, the dried product was dissolved in water and subjected to a second chromatography on Sephadex LH-20.

Example 2

Purification and characterization of AR

AR was prepared as in Example 1, the product eluting as the first peak (Fig. 1), having the absorption maximum at 290 nm, was dried from methanol. The elemental analysis of the compound was carried out by SGS Cervac Wolff (France) and the composition was determined in the Department of Chemistry of Ben Gurion University, Israel, to provide the empiric formula of the product: $Ru_2N_3Cl_5.5H_2O$ (H_2O, HCl).

Example 3

Inhibition of Ca^{2+}-pump activity

The effect of azido-ruthenium on Ca^{2+}-dependent proteins and its photoactivation was demonstrated by the inhibition of the Ca^{2+}-pump activity involved in muscle relaxation as shown in Fig. 5. The preparation of SR membranes from skeletal muscle, and the measurement of Ca^{2+}-accumulation were carried out as described above. The sensitivity of the Ca^{2+}-pump activity to azido-ruthenium was increased more than two-fold by photoactivation, demonstrating that azido-ruthenium of this invention is a photoreactive compound. Figure 5 shows that the inhibition is dependent on time of UV irradiation, as well as on the concentration of the

reagent. UV irradiation increases the inhibitory effect of the compound on Ca^{2+}-accumulation by SR membranes, confirming the photoactivation, and suggesting irreversible binding of the reagent to the protein.

Example 4

Specific inhibition of Ca^{2+} transport in mitochondria

The effect of AR on mitochondrial process of electron transport from the donor succinate to the acceptor cytchrome c was characterized as described above. The effect of azido-ruthenium on the Ca^{2+} uptake, and on the electron transport in mitochondria is shown in Fig. 6. Although said electron transfer involves several dozens electron transfer carriers, this reaction was not affected by azido-ruthenium of this invention. On the other hand, Ca^{2+} transport, which is carried out by a yet unidentified uniporter protein, was inhibited by azido-ruthenium, demonstrating the specificity of azido-ruthenium of this invention.

Example 5

Inhibition of ryanodine binding to SR membranes by AR

The effect of azido-ruthenium on the binding of the toxic alkaloid ryanodine to its receptor, known as the Ca^{2+}-release channel that possesses regulatory Ca^{2+}-binding sites, was measured as described above. Azido-ruthenium of this invention strongly inhibited the binding of ryanodine to SR membranes, as demonstrated in Fig. 7.

Example 6

VDAC channel closure

VDAC is a channel protein which transports Ca^{2+} across the mitochondrial outer membrane. It possesses Ca^{2+}-binding sites [Gincel D. et al.: Biochem J. 358 (2001) 147-55] and thus is involved in regulation of its own activity. Azido-ruthenium inhibited the channel activity of VDAC. Purified VDAC was reconstituted into a planar lipid bilayer as multichannels. The channel activity was measured as the ions passed the current across the bilayer in response to a voltage gradient of 10 mV, as a function of time. In the absence of azido-ruthenium, the channels are open and remain open for up to 2 hours. However, in the presence of azido-ruthenium, most of the channels are closed, as shown in Fig. 8.

Example 7

Specific inhibition of Ca^{2+}- dependent reactions by AR

The specificity of the binding of AR to calcium binding proteins is demonstrated by testing its effects on the activity of different enzymes. The results, summarized in Table 1, indicate that the activities of calcium-independent proteins such as glutamate dehydrogenase, lactate dehydrogenase and alkaline phosphatase were only slightly inhibited by the reagent (less than 10%). In contrast, the activities of calcium-dependent proteins were strongly inhibited by azido-ruthenium (up to 90%). The inhibition was observed for various proteins with different

activities. Such proteins include, for example, proteins which catalyze the transport of Ca^{2+} across the membrane by distinct mechanisms, all of which involve Ca^{2+}-binding to the protein, like the Ca^{2+}-pump that transports Ca^{2+} at the expense of ATP hydrolysis, and the uniporter carrier protein which transports Ca^{2+} accumulated in the mitochondrial matrix as a result of the membrane potential established by the electron transport chain.

Another inhibited activity is that of the ryanodine receptor, Ca^{2+}-release channel, which possesses regulatory Ca^{2+} binding sites that control its channel activity and its ability to bind the toxic alkaloid ryanodine. The results also indicate that azido-ruthenium may interact with Mg^{2+}-dependent proteins, such as hexokinase and pyruvate kinase, but the inhibition is weaker than with Ca^{2+}-binding proteins (only 20-30% of inhibition).

Table 1 Inhibition of enzymes by AR.

Enzymatic Reaction	Divalent cation	AR (µg/ml)	Activity (% of control)
Glutamate Dehydrogenase	None	2.4 6 12	95 90 83
Lactate Dehydrogenase	None	2.4 6 12	96 90 92
Alkaline Phosphatase	None	2.4 6 12	98 92 86
G6P dehydrogenase	None	2.4 6 12	96 102 105
Electron Transport	None	2.4 6 12	96 83 82
Hexokinase	Mg^{2+}	2.4 6 12	85 73 70
Pyruvate Kinase	Mg^{2+}	2.4 6 12	97 89 81
Ca^{2+}-ATPase Skeletal Muscle	Ca^{2+}	2.4 6 12	54 38 24
Ca^{2+}-uptake Skeletal muscle	Ca^{2+}	2.4 6 12	93 61 13
Ca^{2+}-uptake Mitochondria	Ca^{2+}	2.4 6 12	34 29 16
Ca^{2+}- release channel (RyR)	Ca^{2+}	2.4 6 12	49 36 26

Example 8

Identification of unknown Ca^{2+}-binding proteins

By using radiolabeled [^{103}Ru]azido-ruthenium, it is possible to identify Ca^{2+}-binding proteins in a biological sample such as whole cells, isolated mitochondria, ER, or other protein containing fractions. Two crude protein mixtures, crude SR proteins and crude mitochondria proteins, can be submitted to SDS gel electrophoresis and Coomassie staining, as illustrated by Fig. 9 A. Said two crude protein mixtures can be mixed with [^{103}Ru]-labeled AR and UV-irradiated, causing [^{103}Ru]AR to bind covalently to the protein. An X-ray film can then be exposed to the dried gel (autoradiography), which will allow the identification of the [^{103}Ru] radiolabeled proteins, as illustrated in Fig. 9B. The arrows in Fig. 9 indicate known Ca^{2+} -binding proteins in SR and hypothetical proteins in mitochondria. The labeled protein bands can be cut from the gel and subjected to MALDI-TOF analysis and the identity of the protein can be determined by a sequence homology search.

Example 9

Affinity chromatography (Afch)

The specific reversible interaction between a Ca^{2+}-binding protein and azido-ruthenium can be exploited in separation of calcium binding proteins by affinity chromatography (Fig.10). Azido-ruthenium of this invention can be coupled to an inert support such as agarose,

polyacrylamide or polystyrene by photoactivation, for example cellulose or Sepharose^{R} beads can be used for said covalent attachment. From the proteins applied to the column, only Ca^{2+}-binding proteins interact with the ruthenium bound in the column, and are retained. The retained proteins can be eluted with a Ca^{2+}-containing buffer. Proteins eluted with Ca^{2+} represent Ca^{2+}-binding proteins. The proteins can be sequenced and the sequences can be used for the sequence homology search and identification of the proteins.

Example 10

Purification of Ca^{2+}-binding proteins using Afch on AR column.

AR, 4.5 mg, obtained as described in Examples 1 and 2, was incubated with cellulose fibers (Sigma-Aldrich), 1.25 g, in the presence of 10 mM carbonate buffer, pH 7.5, irradiated by UV light for 7 times 2 minutes with short intervals between, and left overnight. The unbound AR was removed by washing with said buffer to provide AR-cellulose. The protein preparations were applied to the column, and, after washing with said buffer, eluted from the column with said buffer further containing 2-20 mM $CaCl_2$.

Rat liver mitochondria, rabbit sarcoplasmic reticulum, rat brain extract, calsequestrin, calmodulin, and troponin were isolated as described above. In order to solubilize the rat liver mitochondrial membrane proteins,

mitochondria were treated with 3% Triton X-100, 2 % C12E9 or 3% Triton X-114. To some of the eluted proteins, either EGTA (2mM) or $CaCl_2$ (2mM) was added, and the samples were subjected to SDS-PAGE (Fig. 11). It can be seen that $CaCl_2$ shifts the electrophoretic mobility of the proteins, which was demonstrated for some known Ca^{2+}-binding proteins [Gregersen H.J. et al.: Adv. Exp. Med. Biol. 269 (1990) 89-92].

Among several dozens of proteins applied to the column, only few proteins were bound to the column and were eluted with $CaCl_2$. Some of the proteins that were eluted from the column with the $CaCl_2$ - containing buffer were identified by MALDI-TOF analysis and are presented in Table 2. The results clearly indicate the specificity of the AR-cellulose column to Ca^{2+}-binding proteins.

Table 2.

Preparation applied	Protein MW (kDa)	Identification by MALDI LC/MS	Metal binding
Rat liver mitochondria	69	Albumin precursor	+
	33	Malate dehydrogenase	+
	45	Aspartate aminotransferase	+
	28	Unknown	+
	36	Unknown	+
Rabbit skeletal muscle sarcoplasmic reticulum	26	Triosephosphate isomerase	+
	49	Enolase	+

	58	Unknown	+
Purified protein	Calsequestrin		+
	Calmodulin		+
	Troponin		+
Rat brain extract	Calbindin		+

Example 11

Development of novel AR-based bio-sensor chips

The specific interaction of AR with Ca^{+}-binding proteins enables to develop a biosensor chip for real-time monitoring of macromolecular interactions with Ca^{2+}. It can be an optical biosensor using surface plasmon resonance (SPR). In principle, the SPR-based technique detects mass and has been designed for the study of biomolecular binding and therefore provides an invaluable tool for proteomics studies of the relationship between protein structure and function. The AR-based bio-sensor chip is an optical biosensor that contains AR interacting specifically with Ca^{2+}-binding proteins. Some of the potential applications include:

i) Identification of new Ca^{2+}-binding proteins. By applying protein containing sample to the chip; only Ca^{2+}-binding proteins would interact with the reagent bound to the chip. Those proteins could be eluted with a Ca^{2+}-containing buffer. Identification of the eluted proteins can be done by the techniques known in the art, such as MALDI-TOF, LC-MS/MS, interactions with antibodies, etc. The chip requires small amounts of the

protein relative to column, and a Ca^{2+}-eluted protein can be obtained within 10 min.

ii) The second chip type allows identification of new protein compounds. Proteins often function as a part of multi-protein compounds; identifying the individual proteins and determining their sites of interaction within the compound are essential for defining their mode of action and function. Chips containing known Ca^{2+}-binding proteins can be used for the identification of other proteins specifically interacting with them.

One of the challenges, facing life sciences researchers today, is bridging the gap between the knowledge of genomic sequence and its protein products at one side, and the understanding of the protein function and cellular behavior at the other side. This invention can contribute to said bridging. A novel chip containing several dozens of known Ca^{2+}-binding proteins can be used in searching for their interacting partners. Binding affinity and kinetics, binding stoichiometry, binding specificity, and epitope mapping can be obtained by charging the chip with known Ca^{2+}-binding proteins and studying their interaction with known proteins. Alternatively, the protein-charged chip can be used to search for unknown interacting protein. In this case the chip will be exposed to potential protein candidates. To identify the interacting protein, the chip will be exposed to Ca^{2+} or EGTA to dissociate said interacting protein from the chip, and the protein will be subjected to analysis by known methods, such

as MALDI-TOF, LC-MS/MS, etc. The invention, in one of its aspects, can provide a tool for fast screening of proteins, for example proteins that are involved in cross-talk within the cell or proteins modified in certain pathological conditions, thus adding another general technique to those provided by genomics and proteomics.

While this invention has been described in terms of some specific examples, modifications and variations are possible. It is therefore understood that within the scope of the appended claims, the invention may be realized otherwise than as specifically described.

CLAIMS

1. A photoreactive azido-ruthenium (AR) compound which binds to a calcium-binding protein.

2. The compound of claim 1, wherein the molar ratio between ruthenium and azide in said compound is 2:1.

3. The compound of claim 1 or claim 2, comprising in its molecule ruthenium, azido group, and chlorine.

4. The compound of claim 3, wherein the molar ratio $Ru:N_3:Cl$ is 2:1:5.

5. The compound of any one of claims 2 to 4, further comprising bound water molecules.

6. The compound of any one of claims 1 to 5, in which one of the atoms is a radioactive isotope.

7. The compound of claim 6, wherein the isotope is ^{103}Ru.

8. The compound of any one of claims 1 to 7, which compound covalently binds to said calcium-binding protein following photo-activation by UV irradiation.

9. The compound of claim 8, which binds to the calcium binding site of said calcium-binding protein.

10. The compound of any one of claims 1 to 9, which specifically binds to said calcium-binding protein, thereby inhibiting its calcium-binding activity.

11. The compound of any one of claims 1 to 10, wherein said calcium-binding protein is selected from the group consisting of proteins involved in signal transduction, muscle contraction, neurotransmitter release, hormone secretion, cell motility, apoptosis, fertilization, cell proliferation, cell mitosis and in gene expression; proteins associated with Ca^{2+}-transport, Ca^{2+}-pumps, and with the mitochondrial uniporter; channel protein VDAC; Ca^{2+}-release channel/ryanodine receptor; IP_3 receptor proteins involved in Ca^{2+}-efflux in mitochondria; and soluble Ca^{2+} binding proteins regulating various cellular activities.

12. The compound of claim 10 or 11, wherein the inhibition of the calcium-binding activity of said calcium-binding protein increases by photo-activation, compared to the inhibition without photo-activation.

13. A method of isolating a calcium-binding protein from a source comprising the same, which method comprises the steps of:

i) providing a source comprising a Ca^{2+}-binding protein;

ii) reacting said source with an AR compound of any one of claims 1 to 12, optionally under photo-activation by UV irradiation,

whereby said calcium-binding protein is bound to said compound;

iii) isolating the material bound to said compound obtained in step (ii); and

iv) releasing the calcium-binding protein from the product obtained in step (iii).

14. A method according to claim 13, wherein the calcium-binding protein is isolated by affinity chromatography.

15. A method according to claim 14, wherein said AR compound is bound to particles of porous polymer that are packed in a column, and wherein any calcium-binding protein is retained in said column, while other proteins are eluted.

16. A method according to claim 14 or 15, wherein said retained calcium-binding proteins are released from the column by calcium ions.

17. A method according to claim 15 or 16, wherein said particles comprise agarose, cellulose, or dextran.

18. A method for characterizing the structure of a calcium-binding protein, wherein said calcium-binding protein has been isolated by a method according to any one of claims 13 to 17, further comprising a method selected from the group consisting of electrophoresis,

autoradiography, liquid chromatography, MALDI-TOF analysis, LC-MS/MS, protein sequencing and a sequence homology search.

19. A method for the preparation of a AR comprising bio-sensor chip comprising the steps of:

    i) providing an azido-ruthenium compound comprising in its molecule ruthenium, azido group, and chlorine at a molar ratio of 2:1:5;

    ii) binding said compound to a polymer such as dextran and coupling it to a suitable support, preferably gold-plated surface to give a chip; and

    iii) optionally stabilizing the resulting chip.

20. Use of the chip of claim 19 for the isolation of calcium binding proteins, comprising :

    i) exposing said chip to a biological sample comprising a calcium binding protein for a time sufficient for binding of the protein with the support-bound ruthenium compound to occur; and

    ii) washing said chip with a buffer comprising either calcium or EGTA.

21. Use of the chip of claim 20, comprising surface plasmon resonance.

22. A method of screening for a calcium-binding substances, preferably calcium binding proteins, comprising the steps of:

i) providing test substances, preferably proteins;

ii) contacting said substances with a ruthenium compound as defined in any one of claims 1 to 12 under conditions which allow binding to occur, preferably under UV irradiation;

iii) isolating from the reaction of (ii) those substances that specifically bind to said ruthenium compound; and

iv) releasing the substances obtained in step (iii) from the ruthenium compound by suitable means.

23. The method of claim 22 further comprising the step of testing the substances obtained in step (iv) for their calcium binding activity.

24. A method according to any one of claims 13 to 18, wherein said AR compound is labeled.

25. A method according to claim 24, wherein said labeling is radioactive labeling.

26. A process for preparing a photoreactive azido-ruthenium compound which binds to a calcium-binding protein, wherein the molar ratio between ruthenium and azide in said compound is 2:1, comprising:

i) reacting in the dark sodium azide with ruthenium (III) chloride in the presence of HCl;

ii) applying the reaction mixture of the previous step onto a chromatographic column selected from cation-exchanger or hydrophobic;

iii) collecting the fractions having high absorbance at 290 nm; and optionally

iv) drying the collected fractions, redissolving them, rechromatographing them, and optionally crystallizing said compound from methanol.

27. The process of claim 26, wherein the HCl has the concentration in the range from 0.5 mol/l to 2 mol/l.

28. The process of claim 26, wherein said sodium azide and ruthenium chloride are reacted at about 100°C for about 2 to 4 hrs.

29. The process of claim 26, wherein the product of AR migrates as a single spot with Rf being about 0.9 during TLC on cellulose F plates, using 0.16 M ammonium formate, pH 8.5 and 20% methanol as the developer.

30. The process of claim 26, wherein said product is soluble in water, DMF and DMSO, less soluble in methanol, and insoluble in ethanol, ether, chloroform, ethyl acetate, n-butanol, and isopropyl alcohol.

31. The process of claim 26, wherein said product has an absorbance maximum at about 290 nm.

32. The process of claim 26, wherein said product has absorbance of about from 250 to 450 in a water solution, in the concentration of 1 mg/ml.

33. Use of an AR compound of any one of claims 1 to 12 in diagnosing a disorder associated with a defect in the function of a Ca^{2+}-binding protein in a subject, comprising:

i) providing a sample of said subject and a control sample of a normal subject;

ii) contacting said samples with an azido-ruthenium compound as defined in any one of claims 1 to 12 under conditions suitable for binding to occur, preferably under UV irradiation;

iii) isolating from the mixtures obtained in ii) ruthenium-bound substances; and

iv) comparing the said substances obtained in iii) for said sample with the substances obtained in step iii) for said control sample;

whereby a difference between the substances obtained in said sample and said control sample indicates a possible disorder in calcium binding proteins in said patient.

34. Use of an AR compound of any one of claims 1 to 12 in the preparation of a medicament for treating a disorder associated with a defect in the function of a Ca^{2+}-binding protein.

35. A pharmaceutical composition containing an AR compound, or a solvate thereof, prepared by a process of any one of claims 26 to 32.

36. A pharmaceutical composition according to claim 35, further comprising a carrier, stabilizer, adjuvant, diluent, or excipient.

37. A pharmaceutical composition according to claim 35, for use as a medicament for treating or preventing a disorder associated with a defect in the function of a Ca^{2+}-binding protein.

38. A pharmaceutical composition according to claim 35, for inhibiting the calcium-binding activity of said Ca^{2+}-binding protein.

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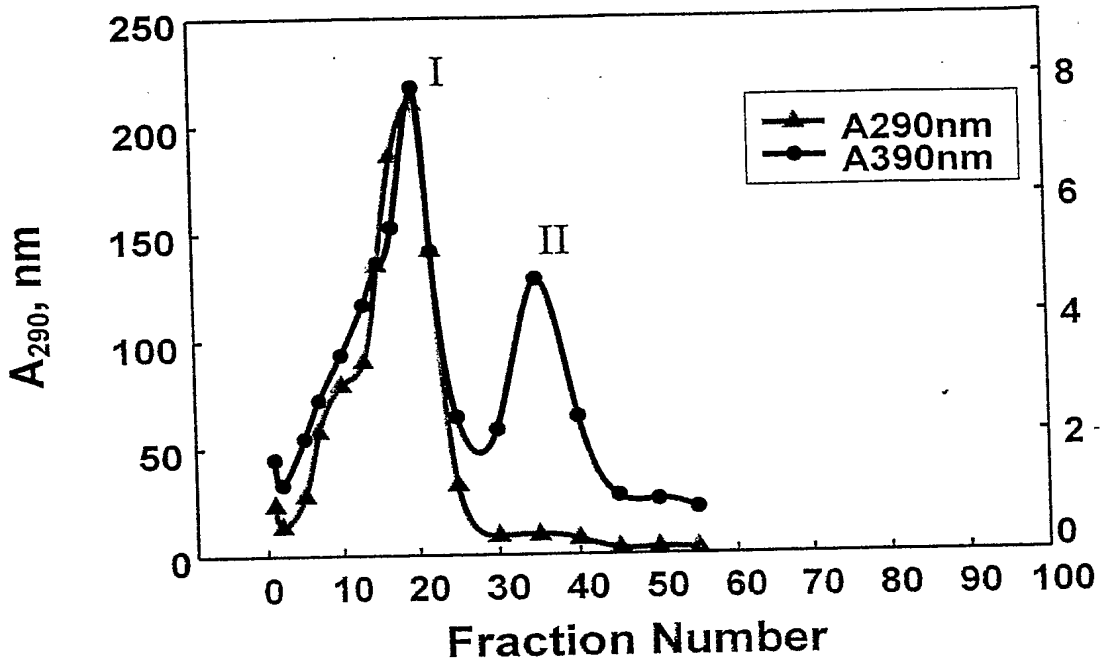
LUZZATTO & LUZZATTO
By עו"ד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100
50
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A390nm
8
6
4
2
0
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Fraction Number

Fig. 1

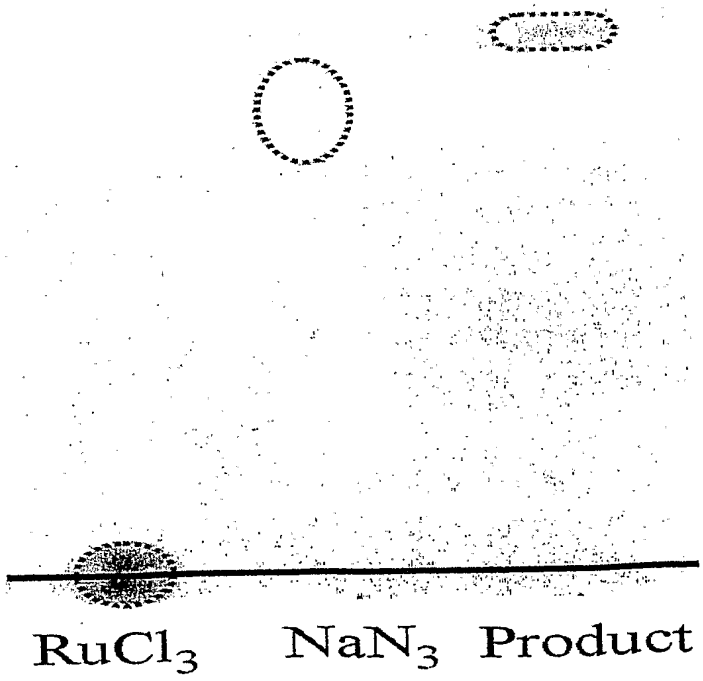
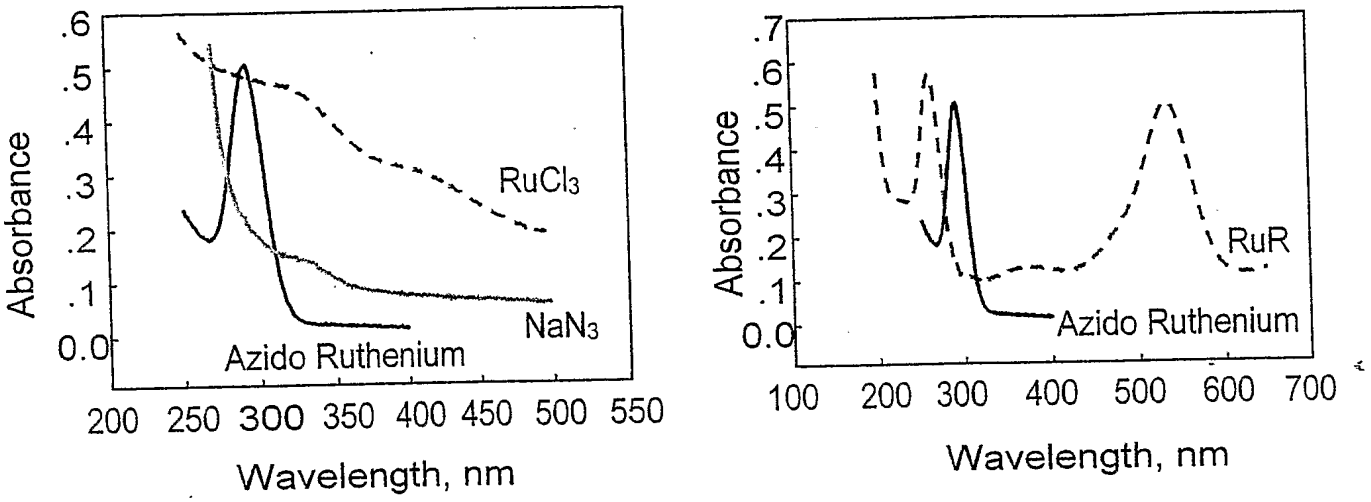

$RuCl_3$
NaN_3
Product

Fig. 2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.5
.4
.3
.2
.1
0.0
RuCl3
NaN3
Azido Ruthenium
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Wavelength, nm
Absorbance
.7
.6
.5
.4
.3
.2
.1
0.0
RuR
Azido Ruthenium
100 200 300 400 500 600 700
Wavelength, nm

Fig. 3

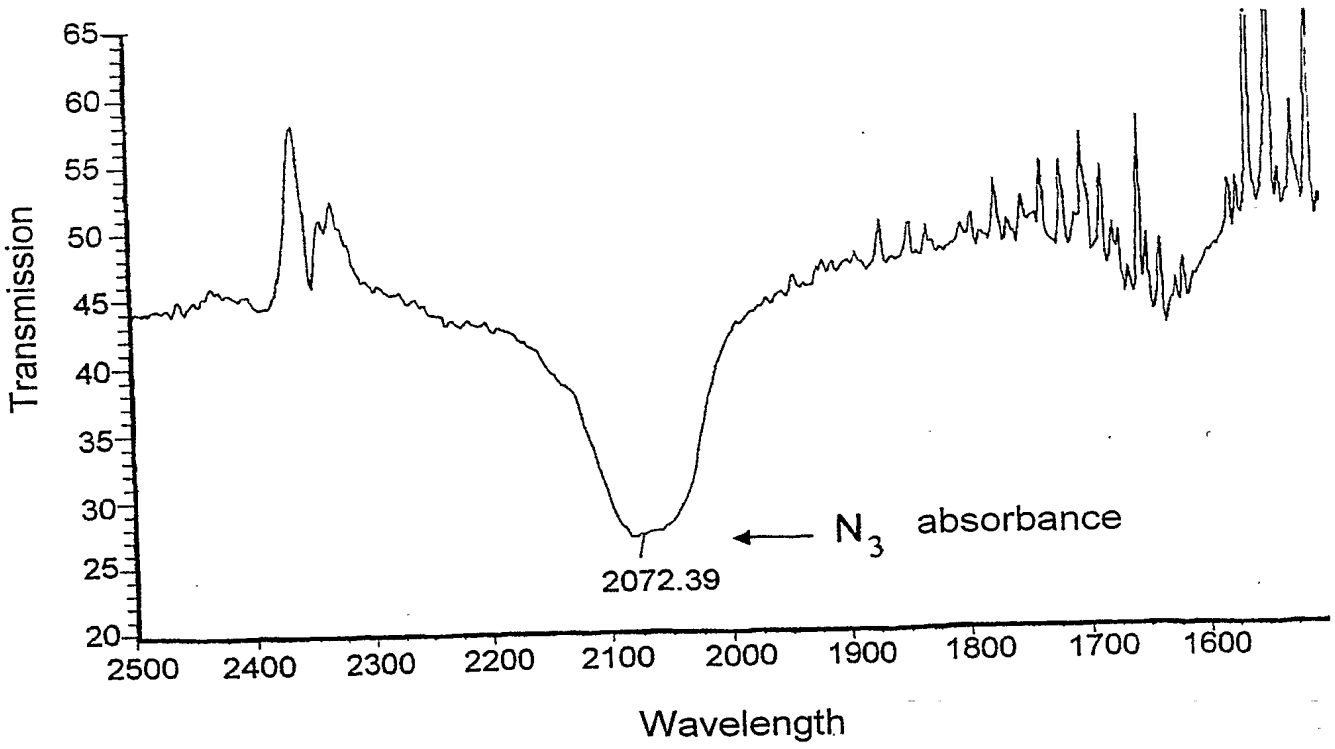

Transmission
65
60
55
50
45
40
35
30
25
20
2072.39
N3 absorbance
2500 2400 2300 2200 2100 2000 1900 1800 1700 1600
Wavelength

Fig. 4

Fig. 5

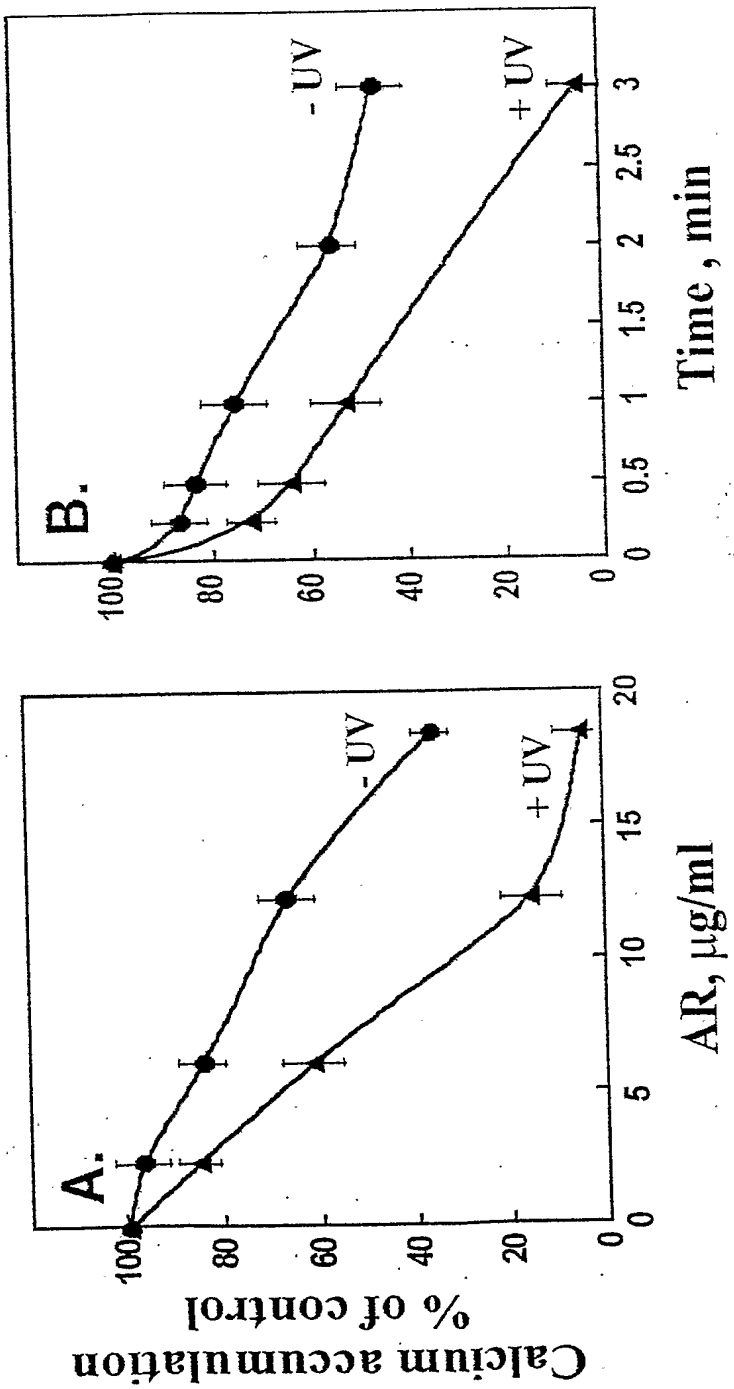
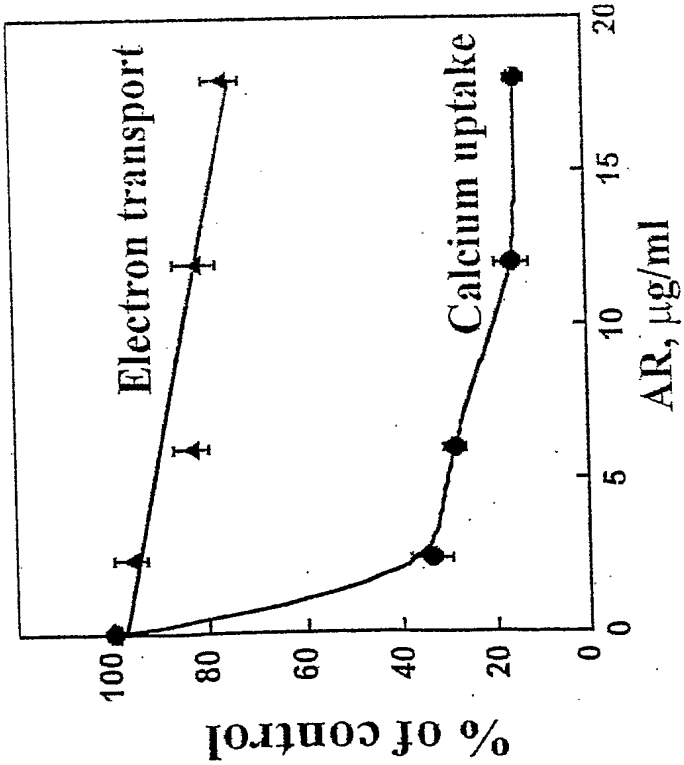

A.
B.
- UV
+ UV
Calcium accumulation
% of control
AR, μg/ml
Time, min

Fig. 6


Electron transport
Calcium uptake
% of control
AR, μg/ml

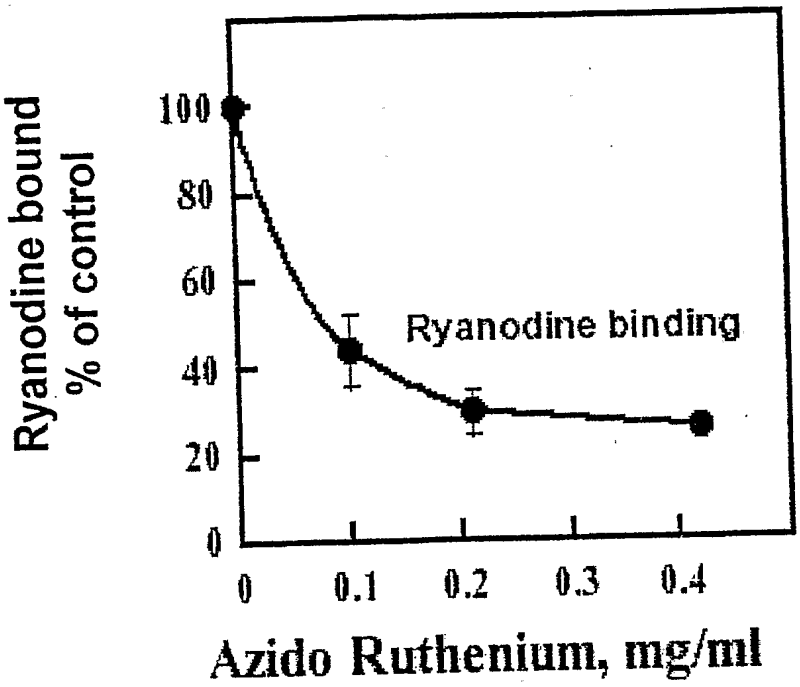
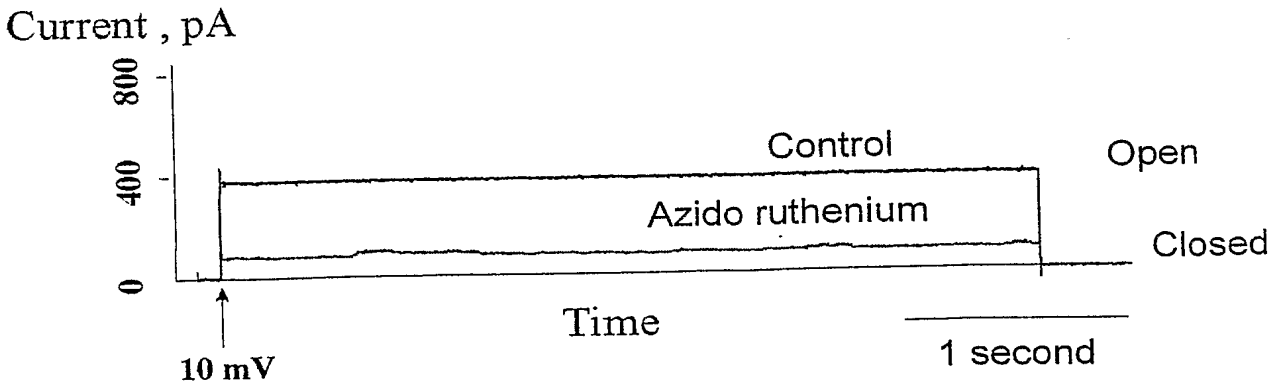

Ryanodine bound
% of control
100
80
60
40
20
0
Ryanodine binding
0
0.1
0.2
0.3
0.4
Azido Ruthenium, mg/ml

Fig. 7


Current , pA
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400
0
Control
Azido ruthenium
Open
Closed
Time
1 second
10 mV

Fig. 8

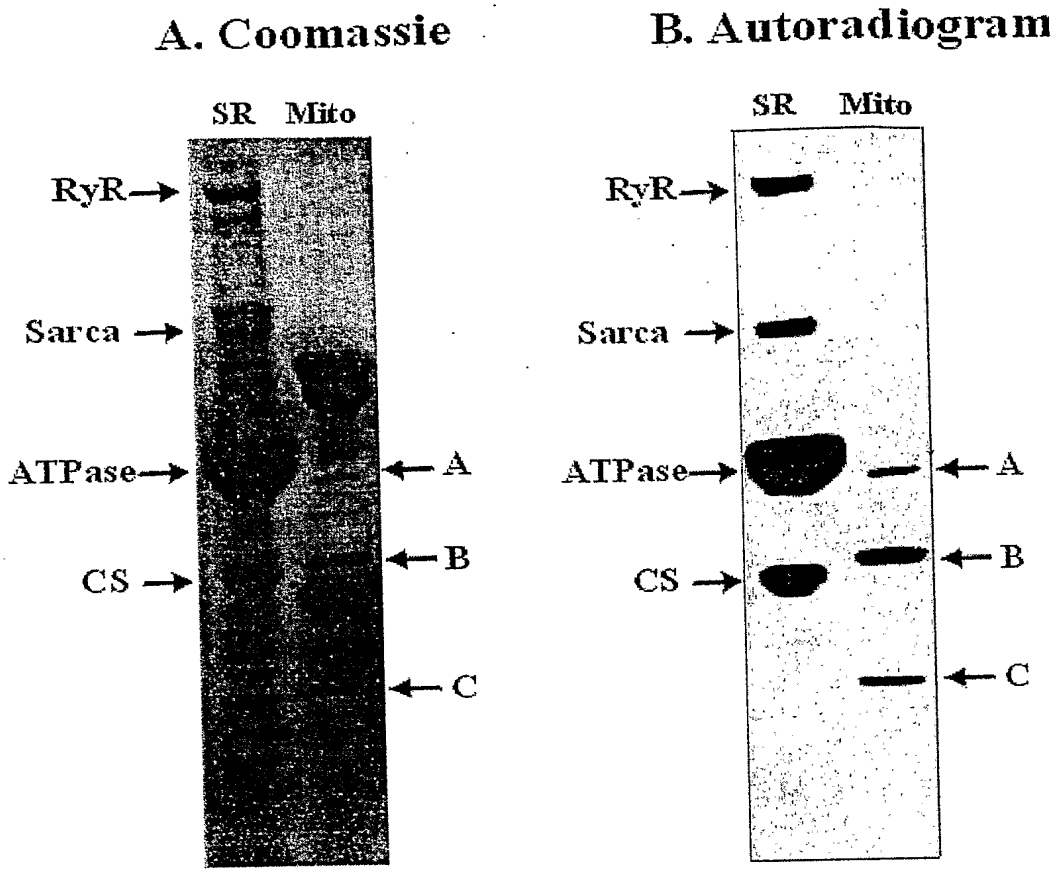

A. Coomassie
B. Autoradiogram
SR
Mito
RyR
Sarca
ATPase
CS
A
B
C

Fig. 9

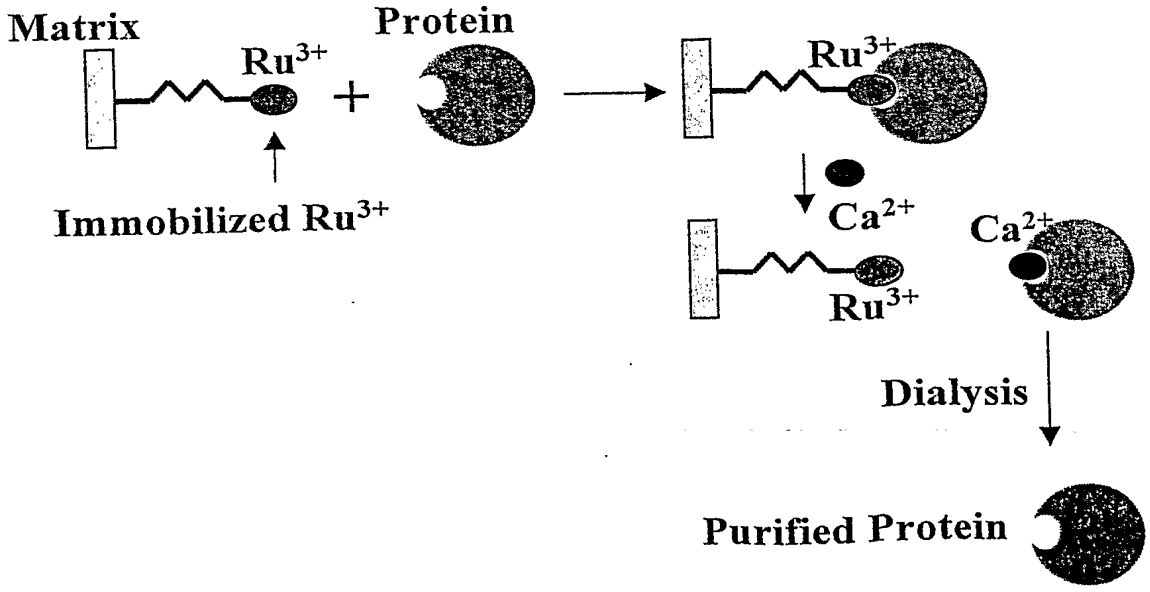
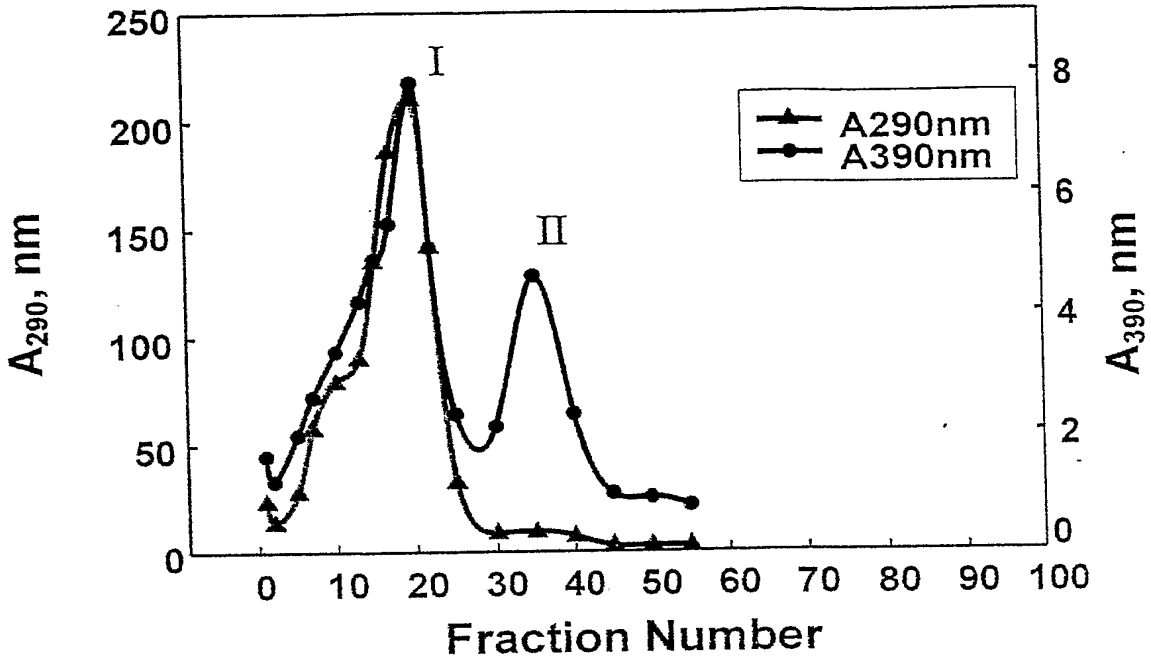

Matrix
Protein
Ru3+
Immobilized Ru3+
Ca2+
Dialysis
Purified Protein

Fig. 10


250
200
150
100
50
0
I
II
A290nm
A390nm
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6
4
2
0
A_{290}, nm
A_{390}, nm
0 10 20 30 40 50 60 70 80 90 100
Fraction Number

Fig. 11

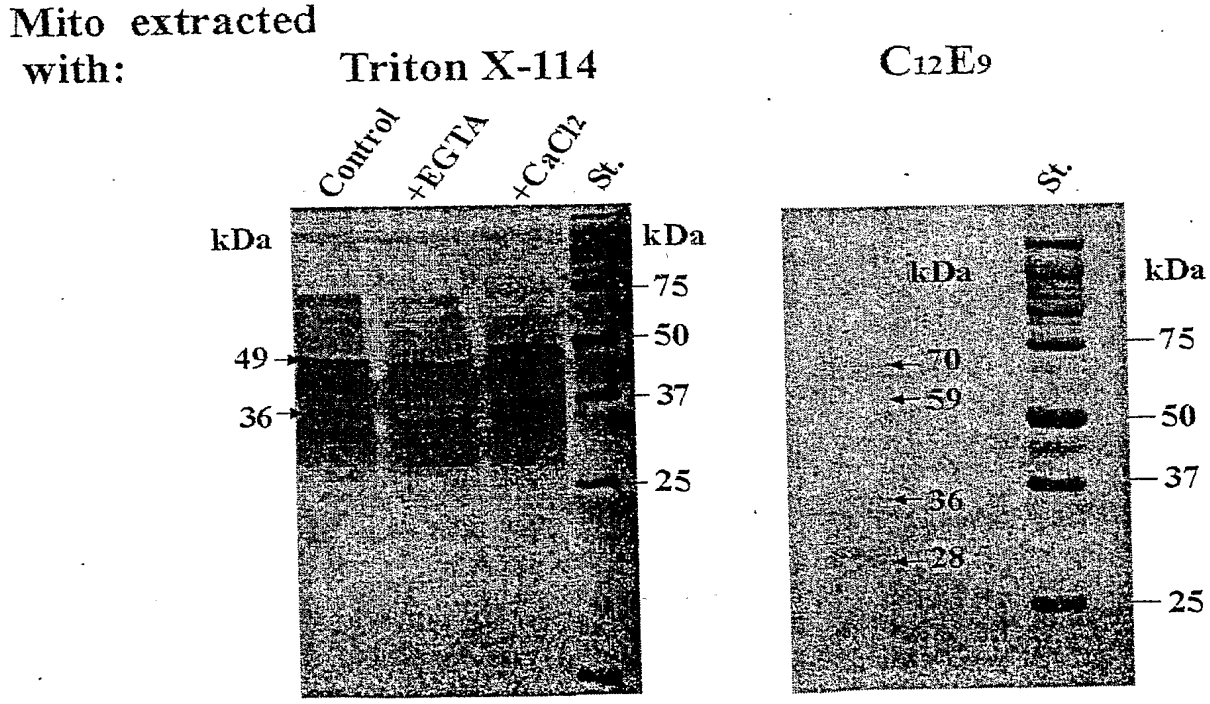
Mito extracted with:
Triton X-114
$C_{12}E_9$
Control
+EGTA
+$CaCl_2$
St.
kDa
75
50
49
37
36
25
St.
kDa
75
70
59
50
37
36
28
25

Fig. 12